Determination of Chloramphenicol in Fish Meat by Liquid Chromatograph-Atmospheric Pressure Photo Ionization-Mass Spectrometry (LC-APPI-MS)

Application

Foods, Environmental

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Abstract

A liquid chromatography-atmospheric pressure photoionization-mass spectrometry method was developed for the determination of chloramphenicol antibiotics in fish meats. For the optimization of APPI, several ion source parameters were examined. Using the optimized parameters, simple mass spectra and a strong signal corresponding to $[M-H]^-$ was observed. The samples were extracted with ethylacetate and evaporated to dryness followed by a clean-up step using liquid-liquid distribution by acetonitrile and n-hexane. Mean recoveries of chloramphenicol from young yellowtail meat and flatfish meat spiked at 0.1–2 ng/g were 89.3%–102.5% and 87.4%–94.8%, respectively. The limit of detection (signal-to-noise = 3) of the young yellowtail meat and the flatfish meat were 0.27 and 0.10 ng/g.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic, that exhibits activity against a variety of aerobic and anaerobic microorganisms. Its action works through interference with or inhibition of protein synthesis. However, weeks or months of CAP therapy can result in a well-understood and irreversible type bone marrow depression called aplasia or hypolasia. This, in turn, can lead to aplastic anemia and although uncommon, it is often fatal. Because of these health concerns, a joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives has proclaimed that CAP residues in the human food supply are unacceptable [1]. The use of CAP in food products has been banned in EU and U.S.A. However, CAP's broad-spectrum activity, ready availability, and low cost attract its use by some third world countries. Admittedly, whenever CAP is accessible, indiscriminate and illegal use potentially exists. In fact, the presence of CAP has been detected in shrimp imported from China and Vietnam that was intended for human consumption.

Liquid chromatography/mass spectrometry (LC/MS) methods are very useful in analyzing CAP in food because of the high selectivity and sensitivity of MS detection [2-7]. Atmospheric pressure ionization (API) interfaces, represented by atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are commonly used in LC/MS.

Atmospheric pressure photoionization (APPI) is a new ionization technique for LC/MS [8, 9]. The APPI source is based on a high-fluence gas discharge lamp that generates vacuum-ultraviolet (VUV) photons of 10 and 10.6 eV energy. The energy of this discharge lamp is normally greater

than a first ionization potential (IP) of an analyte because many organic compounds have IPs in the range of 7–10 eV. On the other hand, the IPs of the most common LC solvents, which are used as a mobile phase, have higher values (water, IP = 12.6 eV; methanol, IP = 10.8 eV; acetonitrile, IP = 12.2 eV). This provides ionization of many analytes with lower IPs without interference from the mobile phase. To our knowledge, APPI has not yet been applied to residual analysis in food.

This application note describes how parameters affect the ionization efficiency of APPI for the analysis of CAP. In addition, the suitability of LC/MS and liquid-liquid extraction using the APPI technique is evaluated for the determination of CAP in fish meat.

Experimental

Chemicals and Solvents

CAP was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The purity of this compound was greater than 99%. Stock solutions at 1 mg/mL were prepared in methanol, stored in the dark at 4 °C, and diluted to the desired concentrations prior to use. Ammonium acetate, pesticide-grade ethyl acetate, anhydrous sodium sulfate, acetonitrile, HPLC-grade methanol and n-hexane were obtained from Wako Chemical (Osaka, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan). A nylon-type 0.22 µm centrifuge filter was obtained from Toyo Soda (Tokyo, Japan).

Sample Preparation

The samples analyzed (young yellowtail and flatfish) were obtained from a local market. To a centrifuge tube, 5 g fish meat and 5 g anhydrous sodium sulfate were weighed and 10 mL ethyl acetate was added. The mixture was homogenized for 20 s with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Staufen, Germany). After centrifugation for 5 min at 6000 rpm, the supernatant was removed and transferred to a round flask. The extraction step was repeated twice, each with 10 mL ethyl acetate. The combined ethyl acetate extract was then evaporated in a rotary evaporator at 40 °C under vacuum. One mL acetonitrile and 1 mL n-hexane was added to the residue, transferred into a graduated glass stopper reagent bottle, and shaken. The n-hexane phase was discarded. The step was repeated with another 1 mL of n-hexane. Finally, the acetonitrile phase was evaporated to dryness under a stream of dry nitrogen using a heating block at 50 °C, redissolved in 5 mL of a 10% acetonitrile in 10 mM ammonium acetate water solution, and filtered through a 0.22 µm nylon centrifuge filter. The samples were spiked with 0.1–100 ng/mL of CAP after the homogenation step to generate a calibration by LC/APPI-MS selected ion monitoring (SIM).

LC/MS

An Agilent 1100 series LC, consisting of a vacuum solvent degassing unit, a binary high-pressure gradient pump, a standard automatic sample injector, and a column thermostat, was used for the separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. See Table 1. The separation was performed on a 150 × 3 mm id column packed with 5 µm Zorbax Eclipse XDB C18 (Agilent Technologies, Palo Alto, USA). A 15-min linear solvent gradient was used for elution with the mobile phase. Quantitative analysis was carried out using SIM of *m/z* 321 with a dwell time of 500 msec.

The following six parameters were optimized using

Table 1. Instrument Parameters

LC:	**1100 series LC**
Column:	Zorbax Eclipse XDB C18 (150 mm × 3 mm, 5 µm)
Solvent A:	Water with 10 mM ammonium acetate
Solvent B:	Methanol
Dopant:	Acetone at 0.05 mL/min
Gradient:	90/10 A/B 15 min to 70/30 A/B
Column temp:	40 °C
Sample volume:	20 µL
Flow rate:	0.5 mL/min
MS:	**1100 MSD, SL**
Ionization:	APPI (Negative)
Scan range:	*m/z* 100–400 for optimization
SIM ion:	*m/z* 321; $(M\text{-}H)^-$
Drying gas:	Nitrogen, 7 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 V
Capillary:	3500 V
Vaporizer temp:	350 °C

the analytical column with CAP at 100 ng/mL: the voltages for in-source-fragmentation (the fragmentor voltage), the capillary voltage (V_{cap}), the drying gas flow rate, the nebulizer pressure, the mobile phase composition, and the mobile phase flow rate. The ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program. Negative ion mass spectra were acquired over the scan range *m/z* 100–400 using a step size of 0.1 amu and a scan rate of 2 s per scan for the optimization of fragmentor voltage.

Results and Discussion

Optimization of the APPI Parameters

To optimize the APPI conditions, parameters that influence the ionization efficiency were investigated. The drying gas flow, the nebulizer gas pressure, the vaporizer temperature, the capillary voltage, and the mobile phase composition were evaluated under the chromatographic conditions mentioned in the Experimental section by SIM mode using the *m/z* 321 ion as the target ion. It was found that modification of drying gas flow rate and nebulizer gas pressure did not drastically improve the sensitivity of CAP. In addition, the fragmentor voltage was included in optimization because of its compound dependence and its significant effect on the mass spectral response.

Effect of Capillary Voltage

The capillary voltage is applied to the inlet of the capillary and influences the transmission efficiency of the ions through the capillary sampling orifice. To establish the optimum capillary voltage, this parameter was varied from 1000 to 4000 V. As shown in Figure 1, 1500 V was found optimum. A tremendous effect of this parameter on the intensity of CAP was observed in the case where acetone was not used as the dopant. On the other hand, when acetone was introduced into the APPI source as the dopant, the maximum intensity of the ion was found at 3500 V. The intensity found at 3500 V with the dopant was higher than the maximum intensity without the dopant. Based on the above results, the capillary voltage was set at 3500 V with acetone.

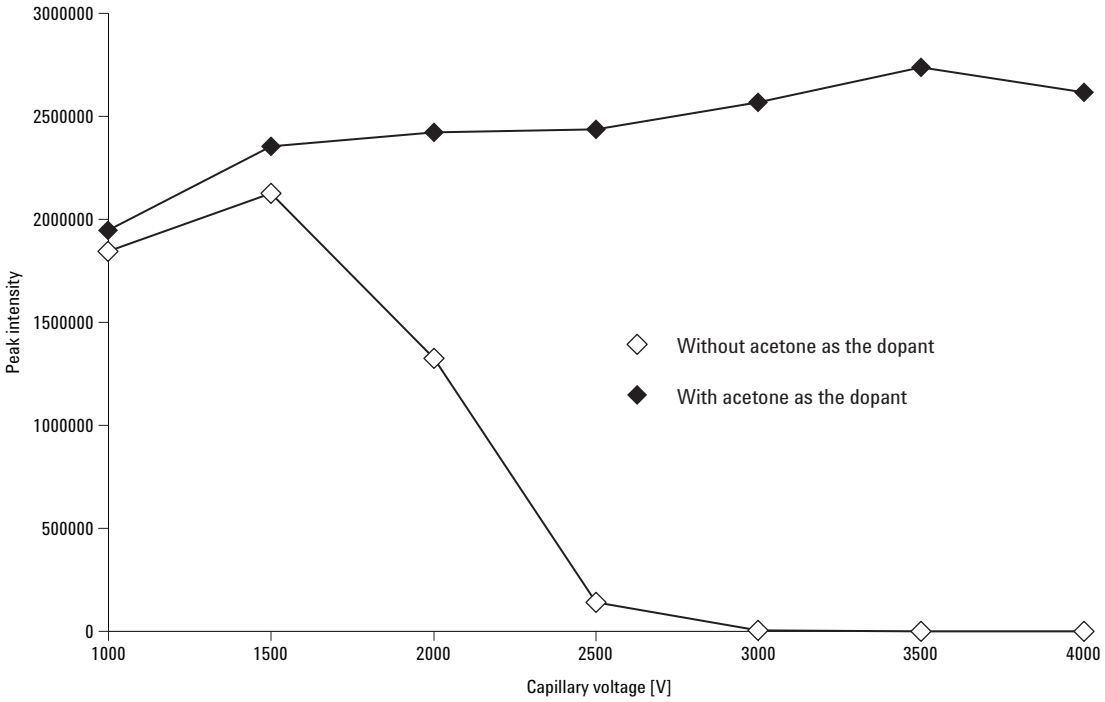

Figure 1. The effect of the capillary voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

Effect of Vaporizer Temperature

In APPI, the vaporizer temperature plays a key role for the complete evaporation of CAP because ionization occurs in the vapor state like APCI. Thus, in the case of using linear gradient elution, this temperature must be kept sufficiently high so that the change of mobile phase composition does not influence the ion intensity of CAP. Under high temperature, however, the risk of thermal degradation occurs. In this study, the vaporizer temperature was modified between 250 and 450 °C to optimize the intensity and the *S/N* ratio. The highest temperature for a maximum intensity and *S/N* ratio of CAP was observed at 350 °C. The intensity of CAP decreased as the vaporizer temperature was increased over 400 °C. In addition, intense fragmentation was observed in the mass spectrum at 400 °C. Therefore, the decrease in intensity above 400 °C seems to be a result of the thermal degradation. Based on the above results, the vaporizer temperature was set at 350 °C.

Optimization of Fragmentor Voltage

The fragmentor voltage is applied to the exit of the capillary and affects the transmission and fragmentation of sample ions between the exit of the capillary and the skimmer at relatively high pressure (3 torr). In general, the higher the fragmentor voltage (which helps the transfer of ions), the more fragmentation will occur. To establish the optimum fragmentor voltage for the analysis of CAP, the intensity of this compound versus the fragmentor voltage was studied in the range from 80 to 200 V. As shown in Figure 2, the optimum fragmentor voltage was found at 120 V, whereas at higher values a significant intensity reduction was observed. Further, the best *S/N* ratio was also observed at 120 V. The mass spectra of CAP at optimal and higher fragmentor voltages are shown in Figure 3. The deprotonated molecule $(M\text{-}H)^-$ was the predominant ion at 120 V, and this included isotopic ions (*m/z* 321, Cl^{35} Cl^{35}; *m/z* 323, Cl^{35} Cl^{37}; *m/z* 325, Cl^{37} Cl^{37}) because CAP includes two

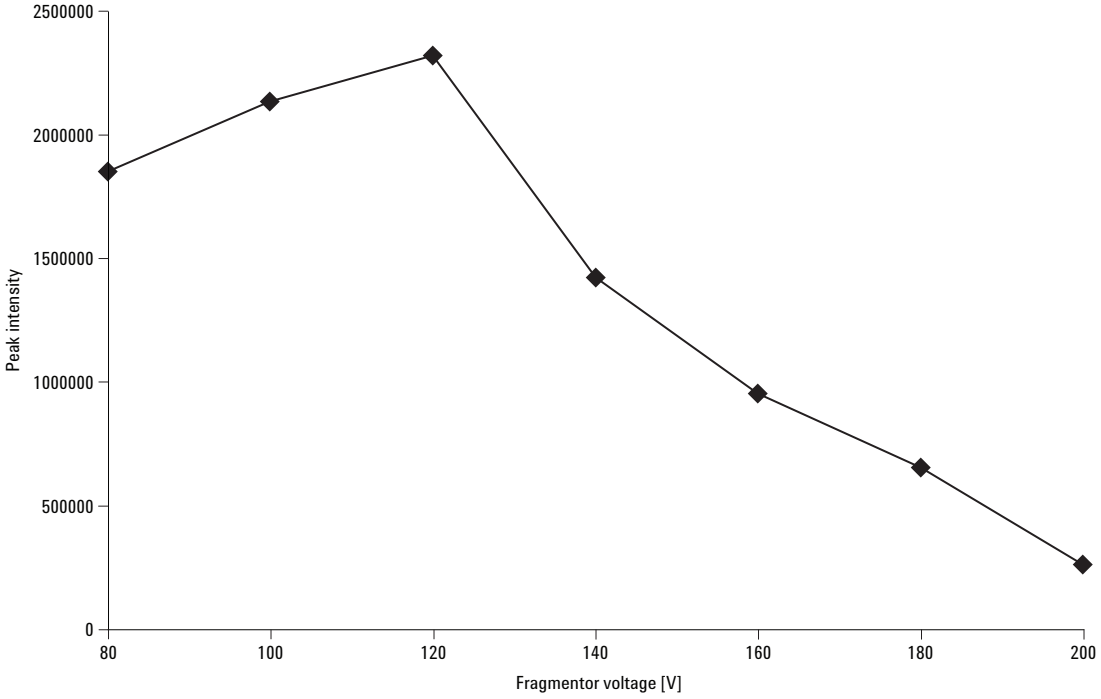

Figure. 2. The effect of the fragmentor voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

chlorines. A higher fragmentor voltage (180 V) generated structurally relevant fragment ions. The *m/z* 152 fragment ion gives the greatest intensity and might be produced by the cleavage of the carbon-carbon bond on the alkyl branch as shown in Figure 3. Other fragment ions are observed at *m/z* 121 and 257. The *m/z* 121 may be the nitrophenyl fragment. The *m/z* 257 fragment might be explained by a charge migration hydrogen shift with a concerted loss of HCl and CO. These observed fragment ions in the APPI source corresponded with the fragment ions in an ESI source and an APCI source. Based on the above results, the fragmentor voltage was set to 120 V.

Optimization of the Chromatographic Conditions

The separation of CAP from sample matrix peaks was optimized using acetonitrile, methanol, water, and ammonium acetate. The combination of methanol and ammonium acetate was found optimum for the separation of CAP. When methanol was replaced with acetonitrile, a significant signal intensity and *S/N* decrease was observed. This result indicates that methanol may be a source of electrons for the hydrogen abstraction from CAP. Therefore, methanol and 10 mM ammonium acetate was used as the mobile phase in this study. The flow rate was set at 0.5 mL/min considering the size of the used column.

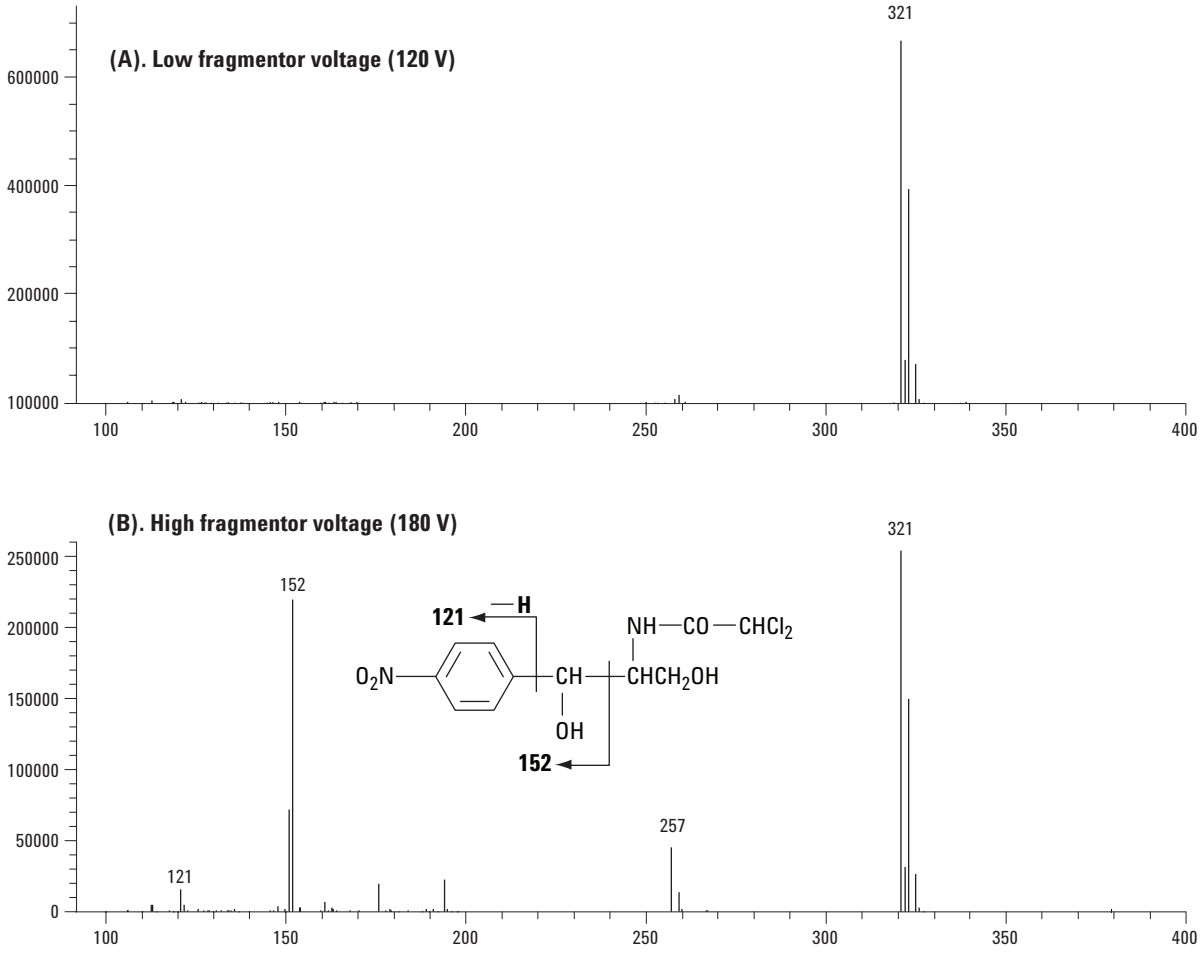

Figure 3. The mass spectra of CAP at two different fragmentor voltages.

Linearity, Detection Limit and Precision of LC/APPI-MS System

The analytical performance characteristics of the optimized LC/APPI-MS were first determined on standard solutions of CAP in pure solvent. See Figure 4. In order to achieve optimum sensitivity, all experiments were carried out under SIM mode using the mass corresponding to the $[M-H]^-$ ions for CAP. To test the linearity of the calibration curves, various concentrations of CAP ranging from 0.1 to 100 ng/mL were analyzed. The calibration curves of APPI showed good linearity with correlation coefficients (r^2) = 0.9998. The repeatability of APPI for a standard solution was calculated on the basis of five replicates at 0.5 ng/mL in the same day. The limit of detection (LOD) was calculated by using a *S/N* ratio of 3 at 0.1 ng/mL. The SIM chromatogram of CAP with APPI is shown in Figure 4 (the *S/N* ratio of this chromatogram was 4.2); LOD and RSD were 0.07 ng/mL and 2.1%.

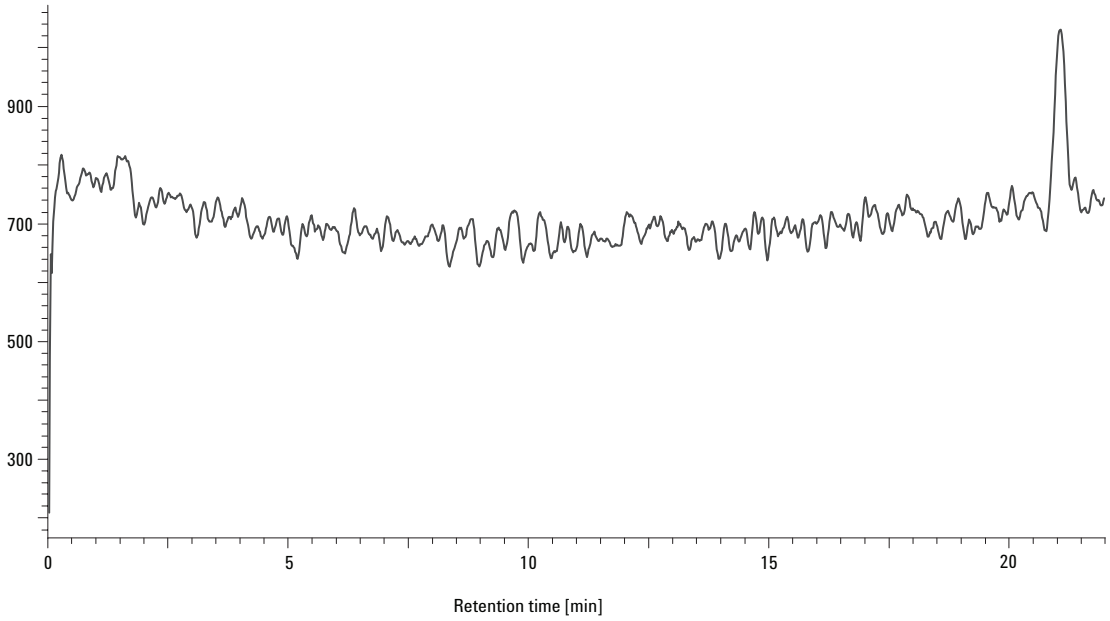

Figure 4. SIM chromatogram of CAP in pure solvent at 0.1 ng/mL with APPI.

APPI Method Evaluation

To evaluate recoveries, the proposed method was applied to the analysis of spiked CAP-free samples of young yellowtail and flatfish meat. Eighteen samples of two different fish were each spiked with CAP and each sample was spiked at three levels. The spiking levels ranged from 0.1 to 2 ng/g. Typical chromatograms from the fish meat extracts spiked at 1 ng/g and 0.1 ng/g are shown in Figure 5.

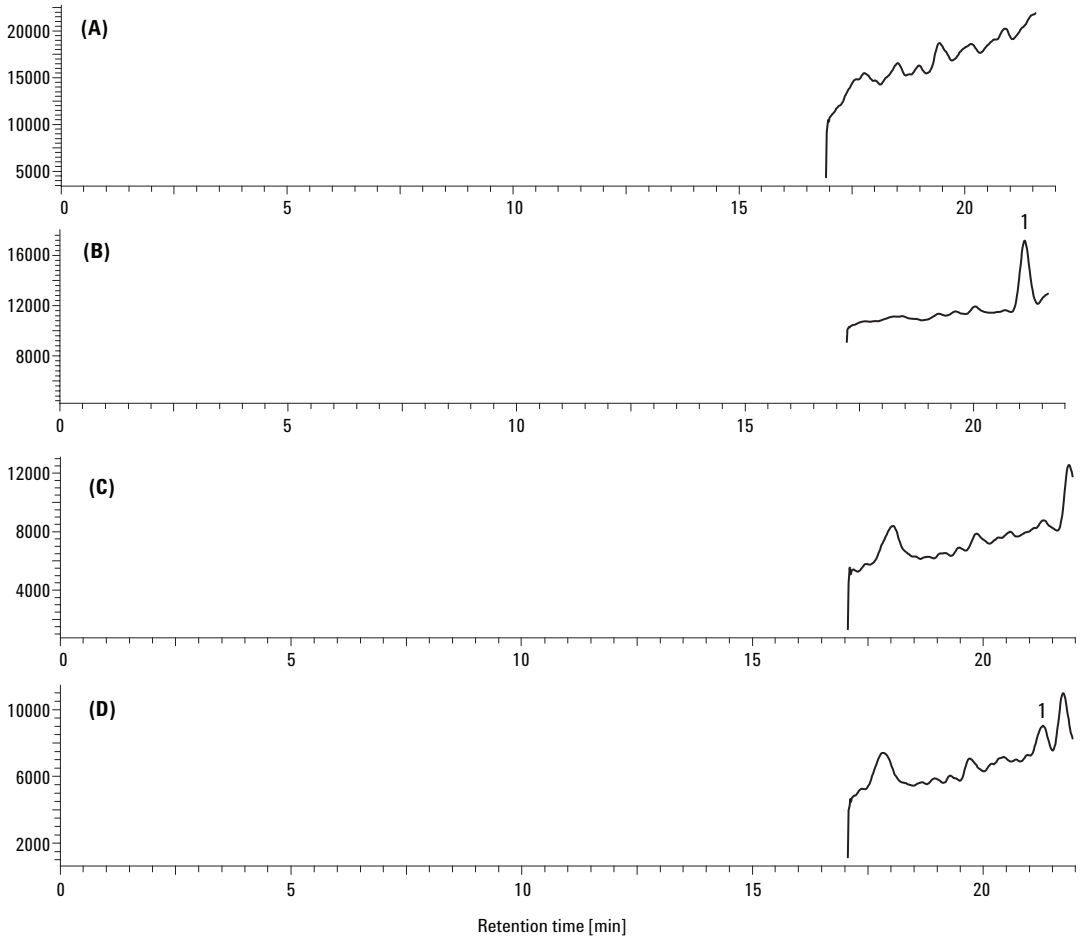

Figure 5. SIM chromatograms of A) Young yellowtail meat, B) Spiked young yellowtail meat at 1 ng/g CAP, C) a flatfish meat, and D) a spiked flatfish meat at 0.1 ng/g CAP.

Data from 18 spiked samples led to recoveries and RSD are summarized in Table 2.

Table 2 Recovery of CAP for Spiked Fish Meat

Spiking levels (ng/g)	Recovery [±RSD (%)]* Young yellowtail	Flatfish
0.1	89.3 ±5.1	87.4 ±6.1
0.5	102.5 ±4.9	94.8 ±6.7
2.0	96.1 ±4.3	91.8 ±4.9

*Three spiked samples at the same amount were analyzed.

Mean recoveries ranged from 87.4% to 102.5% with RSD of 4.3% to 6.7%. The LODs of CAP in fish meats were determined by the signal corresponding to three times the background noise on SIM chromatogram of spiked sample at 1 ng/g and 0.1 ng/g and shown in Table 3. The intraday precision (repeatability) was estimated by injecting the same spiked fish meat extract at 0.1 ng/g five times during a working day. The interday precision (reproducibility) was evaluated by analyzing the same sample over 5 working days. The repeatability and reproducibility for CAP in fish meats were 4.8%, 9.4% and 2.1%, 7.3%, respectively. These results indicate that this LC/APPI-MS method is suitable for the analysis of residues of CAP in fish meats.

Table 3. LODs, Repeatability, and Reproducibility of CAP in Standard Solution Using APPI

Fish meats	LODs* (ng/g)	Repeatability** (RSD, %)	Reproducibility*** (RSD, %)
Young yellowtail	0.27	4.8	9.4
Flatfish	0.10	2.1	7.3

*Detection limit is LOD defined as $S/N = 3$ at 0.1 ng/mL.

**Repeatability was calculated on the basis of five replicates at 0.5 ng/mL within 1 day.

***Reproducibility was calculated by analyzing one fish meat spiked at 0.1 ng mL^{-1} per day during 5 days.

Conclusion

APPI is an ideal ionization technique because of high sensitivity and high selectivity for the determination of CAP in fish meats. An important advantage of using APPI for CAP content of fish meats is that sample matrix did not significantly affect ion intensity of CAP. The data presented here demonstrate that this method is convenient for routine analysis of CAP residues in fish meats at trace levels, as excellent recoveries and precision for different samples were obtained.

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